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**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1652359> since 2017-11-20T15:44:49Z

*Published version:*

DOI:10.23736/S0031-0808.17.03353-5

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# UNIVERSITÀ DEGLI STUDI DI TORINO

***This is an author version of the contribution published on:***

*Questa è la versione dell'autore dell'opera:*

*[Panminerva Medica, 59(4), 2017, doi: 10.23736/S0031-0808.17.03353-5]*

*ovvero [Caviglia GP, Abate ML, Gaia S, Petrini E, Bosco C, Olivero A, Rosso C, Ciano A, Pellicano R, Saracco GM, Rizzetto M, Smedile A., 59, editore Minerva Medica, 2017, pagg.283-289]*

***The definitive version is available at:***

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Original article

**Risk of hepatocellular carcinoma in HBV cirrhotic patients assessed by the combination of miR-122, AFP and PIVKA-II**

Running title: MiR-122+AFP+PIVKA-II and HCC

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## **ABSTRACT**

**BACKGROUND:** Reliable biomarkers for early detection of hepatocellular carcinoma (HCC) in patients with cirrhosis are lacking. We evaluated the use of miR-122, alpha-fetoprotein (AFP) and protein induced by vitamin k absence/antagonist II (PIVKA-II) for HCC risk prediction in patients with HBV-related cirrhosis under surveillance.

**METHODS:** We first analyzed a group of 63 patients with HBV-related liver cirrhosis of whom 33 had HCC. Then we performed a retrospective analysis on another group of 13 cirrhotic patients who developed HCC during surveillance, of whom serial serum samples were available (at time of HCC diagnosis [T<sub>0</sub>], 6-9 months [T<sub>-1</sub>] and 12-18 months [T<sub>-2</sub>] before HCC detection). Serum miR-122 levels were assessed by quantitative real time-PCR, whereas AFP and PIVKA-II were measured by fully automated chemiluminescent enzyme immunoassay.

**RESULTS:** Serum levels of miR-122, AFP and PIVKA-II were different between patients with cirrhosis and those with HCC (P=0.024, P<0.001 and P<0.001, respectively). Areas under the curve (AUC) were 0.675 for miR-122, 0.791 for AFP and 0.846 for PIVKA-II, while their combination improved the discrimination power between cirrhosis and HCC (AUC=0.918). In the longitudinal study, we found a significant variation overtime for the biomarkers combination (P=0.011) but not for each single biomarker (miR-122, P=0.163; AFP, P=0.170; PIVKA-II, P=0.447). Combined miR-122+AFP+PIVKA-II adjusted Hazard Ratio for HCC development was 10.63, 95% confidence interval 1.87-60.28 (P<0.001).

**CONCLUSIONS:** In HBV-related cirrhosis, the combination of miR-122, AFP and PIVKA-II enables the identification of patients at higher risk of HCC development that could benefit from closer monitoring.

**Key words:** Alpha-fetoprotein - Hepatocellular carcinoma - MicroRNA - Protein induced by vitamin k  
absence or antagonist II - Surveillance

The natural history of chronic hepatitis B virus (HBV) infection is characterized by a chronic inflammation that leads to fibrosis progression and to cirrhosis, a condition at high risk for hepatocellular carcinoma (HCC) development.<sup>1, 2</sup> Moreover, HBV infection represents an additional risk factor due to the direct oncogenic properties of the virus.<sup>3, 4</sup>

Currently, surveillance programs for HCC detection in high risk population are mainly based on abdominal ultrasonography (US). According to the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases clinical guidelines for HCC management, surveillance should be performed every 6 months by US, while diagnosis should be based on imaging methods, such as computed tomography (CT) and magnetic resonance imaging (MRI), and/or biopsy.<sup>5, 6</sup> Beside imaging methods, the Japan Society of Hepatology and the Asian Pacific Association for the Study of the Liver guidelines suggest the use of serum biomarkers such as alpha-fetoprotein (AFP) and des-gamma-carboxy-prothrombin (DCP), also known as protein induced by vitamin k absence or antagonist II (PIVKA-II), for surveillance programs and for early HCC detection.<sup>7, 8</sup>

Several studies investigating the accuracy for HCC detection of either traditional biomarkers measured by novel highly sensitive methods or novel classes of biomarkers involved in epigenetic machinery (i.e. microRNAs [miRNAs]) reported promising results.<sup>9-11</sup> Moreover, the combination of different biomarkers with demographic or clinical data into scores permitted to further enhance diagnostic accuracy, providing to clinicians potential tools to improve the management of patients at risk of HCC.<sup>12, 13</sup>

In the last decade, the role of miRNAs in HCC and liver disease progression has been intensely investigated.<sup>14-16</sup> In particular miR-122, a liver-specific miRNA that accounts for more than 70% of the total hepatic miRNA pool, has been implicated in different molecular pathways.<sup>17</sup> Recently, it has been

reported that among selected miRNAs, only miR-122 serum levels varied significantly between patients with HBV-HCC and those with HBV-cirrhosis without HCC.<sup>18</sup>

The aim of the present proof-of-concept study was to explore the performance of miR-122, AFP and PIVKA-II, alone or in combination, for HCC prediction in patients with HBV-related cirrhosis on long term follow up.

## **Materials and methods**

### *Study population*

The study involved a cross-sectional and a longitudinal cohort of hepatitis B surface antigen (HBsAg)-positive patients on follow-up between June 2012 and December 2015.

Inclusion criteria were: age between 18 and 80 years, HBsAg-positivity, signed written informed consent. Exclusion criteria included: anti-HCV-positivity, anti-HIV-positivity, alcohol intake >40 g/day, concomitant hepatic comorbidities (i.e. hemochromatosis, Wilson disease, alpha1-antitrypsin deficiency, autoimmunity) and no signed written informed consent. Additional inclusion criteria for the longitudinal study cohort was the availability for each patient of at least 2 serum samples collected within 2 years before HCC diagnosis and stored to -20°C.

The degree of liver disease was assessed according to clinical, biochemical and histological criteria. Liver cirrhosis was diagnosed by liver biopsy or by laboratory data and imaging findings (abdominal US and transient elastography).<sup>19, 20</sup> Diagnosis of HCC was established by CT scan and staging was assessed by BCLC criteria.<sup>5</sup>

The study protocol was compliant to the Declaration of Helsinki and it was approved by the Institutional Ethics Committee (CEI-452).

### *MicroRNAs quantitation*

MiRNAs quantitation was performed by in-house developed real-time RT-PCR assay.

Extraction was performed from 250 µL of serum by miRCURY™ RNA isolation kit - Biofluids (Exiqon A/S, Denmark). RNA was eluted in 50 µL of nuclease-free water and reverse transcribed using miRCURY LNA™ Universal RT - cDNA synthesis kit (Exiqon). The cDNA was amplified in duplicate using miR-specific primers and ExiLent SYBR® Green master mix (Exiqon). Real-Time PCR was performed on a CFX96 thermal cycler (Bio-Rad, USA). To monitor the efficiency of extraction, a synthetic miRNA pool (*Caernohabditis elegans* [cel]-miR-39 and cel-miR-54) was spiked in each sample prior to RNA purification. Serum miRNA levels were calculated from cycle threshold (Ct) using the  $2^{-\Delta Ct}$  method and relative concentrations were expressed in “arbitrary units” (AU).

#### *AFP and PIVKA-II measurement*

Serum levels of AFP and PIVKA-II were determined on a fully automated chemiluminescent enzyme immunoassay (CLEIA) system, Lumipulse® G600 II analyzer (Fujirebio Europe, Gent, Belgium) using Lumipulse G AFP-N and Lumipulse G PIVKA-II reaction cartridges according to manufacturer's instructions. AFP and PIVKA-II concentrations were given in ng/mL and mAU/mL, respectively. Detection limit of AFP and PIVKA-II assays were 0.075 ng/mL and 1.37 mAU/mL, respectively.

#### *Statistical analysis*

MiRNAs, AFP and PIVKA-II values were expressed as median and 95% confidence interval (CI). D'Agostino-Pearson test was used to test data normality. Mann-Whitney test and Fisher's exact test were used to compare continuous and categorical variables, respectively. To evaluate diagnostic performance of miRNAs, AFP, PIVKA-II alone or in combination, area under the curve (AUC), sensitivity (Se), specificity (Sp), positive likelihood ratio (+LR) and negative likelihood ratio (-LR)



were assessed by using receiver operating characteristic (ROC) curves analysis. Predicted probabilities calculated by logistic regression model, including miRNAs, AFP and PIVKA-II as independent variables and HCC presence as dependent variable, were used for ROC analysis in order to evaluate the performance of the combination of the three biomarkers.

Non-parametric Friedman test was used to evaluate biomarkers kinetics. Cox proportional-hazards regression was performed to calculate univariate and multivariate Hazard Ratios (HR) of HCC development. The comparison between survival curves was performed by the Kaplan-Meier method with a log-rank test.

A two-tailed  $P < 0.05$  was considered statistically significant. All statistical analyses were performed using MedCalc software, version 12.7.0.0. (MedCalc, Ostend, Belgium).

## **Results**

### *Cross-sectional analysis*

Overall 63 subjects were included in the cross-sectional cohort: 30 patients with HBV-related cirrhosis (19 male [M], 11 female [F]; median age 54 [50-58] years) and 33 patients with a diagnosis of HBV-related HCC (29 M, 4 F; median age 63 [62-64] years). Patients' characteristics are reported in Table I. Median miR-122, AFP and PIVKA-II serum levels of patients with cirrhosis and those with HCC are depicted in Figure 1.

MiR-122 showed a moderate diagnostic accuracy for the discrimination between cirrhosis and HCC (AUC=0.675). The cut-off that maximized Se and Sp was 35.8 AU (Se=0.61, Sp=0.76, +LR=2.53 and -LR=0.52). Conversely, an higher performance was observed for AFP and PIVKA-II (AUC=0.791 and AUC=0.846, respectively). The cut-off that maximized Se and Sp for the detection of HCC among patients with cirrhosis was 9.5 ng/mL for AFP (Se=0.61, Sp=0.87, +LR=4.70 and -LR=0.45) and 58 mAU/mL for PIVKA-II (Se=0.91, Sp=0.71, +LR=3.13 and -LR=0.13).

The combination of the 3 biomarkers into a model providing probability (P) of HCC, further increased the diagnostic accuracy (AUC=0.918). Mean P (HCC) values were 0.22 (0.15-0.28) in patients with cirrhosis and 0.90 (0.76-1.00) in those with HCC ( $P<0.001$ ). The cut-off that maximized Se and Sp was 0.39 (Se=0.91, Sp=0.88, +LR=7.58 and -LR=0.10) (Figure 2).

### *Longitudinal analysis*

The longitudinal study cohort included 13 HBsAg-positive patients (11 M, 2 F; median age 63 [61-67] years) with cirrhosis under surveillance who developed HCC within the study period (Table I). For all patients, 3 serum samples corresponding to different time-points were analyzed: HCC diagnosis or T<sub>0</sub>, median 7.8 (range 4.9-11.2) months or T<sub>-1</sub> and median 15.0 (range 13.2-19.3) months or T<sub>-2</sub>, before HCC diagnosis. Serum levels of MiR-122, AFP and PIVKA-II were 29.7 (10.0-76.7) AU, 5.5 (4.2-12.7) ng/mL and 68 (60-93) mAU/mL at T<sub>-2</sub>, 15.8 (6.2-63.3) AU, 6.2 (4.6-22.7) ng/mL and 73 (42.1-115.6) mAU/mL at T<sub>-1</sub>, and 13.0 (5.3-94.5) AU, 9.8 (5.4-216.9) ng/mL and 69 (53-240) mAU/mL at T<sub>0</sub>, respectively. No significant variation was observed for each single biomarker (miR-122,  $P=0.163$ ; AFP,  $P=0.170$ ; PIVKA-II,  $P=0.447$ ) over time. Whereas, when we analyzed P(HCC) trend using the 3 biomarkers combination, we obtained a statistically significant variation ( $P=0.011$ ). Mean P (HCC) values were 0.44 (0.34-0.56) at T<sub>-2</sub>, 0.53 (0.24-0.94) at T<sub>-1</sub> and 0.73 (0.48-0.99) at T<sub>0</sub> (Figure 3).

### *Prediction of HCC development*

We found no difference of P (HCC) between cross-sectional and longitudinal (T<sub>0</sub>) HCC patients (0.90 [0.76-1.00] vs. 0.73 [0.48-0.99],  $P=0.087$ ), whereas a significant difference was observed between cross-sectional and longitudinal (both T<sub>-1</sub> and T<sub>-2</sub>) patients with cirrhosis (0.22 [0.15-0.29] vs. 0.53 [0.24-0.94],  $P=0.006$  and 0.22 [0.15-0.29] vs. 0.44 [0.34-0.56],  $P=0.002$ , respectively). Using ROC

curves analysis, we identified a P (HCC) cut off value of 0.30 for the discrimination of patients who will develop HCC (longitudinal group at T<sub>2</sub>) from those who will not (cirrhotic patients of the cross-sectional group, still HCC-free at last follow-up). We obtained significantly different survival curves by means of this cut-off as dichotomous variable ( $P < 0.001$ ) (Figure 4). Multivariate Cox proportional-hazards regression analysis, adjusted for age and gender, showed HR=10.63 (95% CI 1.87-60.28) for HCC development in cirrhotic patients with  $P(\text{HCC}) > 0.30$ .

## Discussion

The use of serum biomarkers for HCC surveillance is still debated due to lack of evidence in improving early HCC detection or patient outcomes.<sup>21</sup> Nevertheless, conventional markers as AFP and PIVKA-II are used in clinical practice, although they are not recommended by scientific guidelines for their suboptimal performance.

In recent years, there has been a growing interest to explore circulating miRNAs as potential biomarkers for early HCC detection.<sup>22</sup> In the present study, carried out in patients with HBV-related liver disease, we found that miR-122 serum levels were significantly lower in patients with HCC compared to those with cirrhosis ( $P = 0.024$ ). This finding is in agreement with the tumor suppressor properties described for this miRNA. Consistently, it has been reported that miR-122 loss in HBV-infected hepatocytes could accelerate tumorigenesis.<sup>22</sup> Moreover, HBx protein could down-regulate miR-122 expression, inducing cell proliferation and release from G1/S arrest in malignant hepatocytes.<sup>23</sup>

Performance of traditional biomarkers has been re-evaluated by highly sensitive CLEIA methods, reporting a moderate-to-good diagnostic accuracy for HCC detection, with PIVKA-II outperforming AFP in almost all studies (AUC=0.718-0.890 and AUC=0.618-0.829 for PIVKA-II and AFP, respectively).<sup>24-28</sup> Accordingly, we found that PIVKA-II was superior to AFP (AUC=0.846 vs.

AUC=0.791, respectively). In addition, it has been previously demonstrated on a large group of patients with cirrhosis that PIVKA-II values >55mAU/mL could predict HCC development up to 18 months before diagnosis (HR=3.71, 95% CI 1.65-8.31, P=0.002).<sup>29</sup>

Several attempts to combine different clinical parameters with biomarkers in order to improve HCC surveillance effectiveness have been reported.<sup>13, 30</sup> Johnson *et al* developed and validated a model involving gender, age, AFP-L3, AFP and DCP ("GALAD model") that showed excellent performance (AUC>0.9) in discriminating patients with chronic liver disease/cirrhosis from those with HCC and this model was also confirmed by our group.<sup>9, 12</sup> In the present study, we found that miR-122, AFP and PIVKA-II combination further enhance diagnostic accuracy for HCC detection (AUC=0.918) with Se and Sp values approaching 0.90, being PIVKA-II the main contributing factor. Furthermore, when we combined these biomarkers into a model for HCC risk assessment in cirrhotic patients under surveillance, we found a 10-fold higher risk of HCC development up to 18 months prior cancer diagnosis in those with P(HCC) values >0.30.

These preliminary results are limited by the small number of patients included in both cross-sectional and longitudinal cohorts. Considering the actual burden of HBV infection in Italy (5-10% HBV-related liver disease) and the annual incidence of HCC among high risk patients (2-5%),<sup>31, 32</sup> these findings foster a multicenter prospective study to validate this model for monitoring changes in probability of HCC development.

## Conclusions

Our data strengthen the added value of using biomarkers combination compared to a single marker for HCC detection. The use of miR-122, AFP and PIVKA-II combination into a model for HCC prediction (P-HCC), could provide a tool to personalize surveillance strategies, thus allowing tumor recognition before imaging discovery.

## **Acknowledgements**

Authors thank Lodovico Terzi for statistical advice and Corinna Orsini (Fujirebio Italia) for supplying PIVKA-II test reagents. Fujirebio Italia had no role in study design, in data collection, analysis and interpretation, in manuscript writing and decision to submit for publication.

*Financial support.* The study was funded by a local research grant of the Department of Medical Sciences, University of Turin (SMEA\_RIC\_LOC\_15\_01).

*Conflict of Interest.* The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

## References

1. Caviglia GP, Abate ML, Pellicano R, Smedile A. Chronic hepatitis B therapy: available drugs and treatment guidelines. *Minerva Gastroenterol Dietol* 2015;61:61-70.
2. Dandri M, Petersen J. Latest developments in the treatment of hepatitis B. *Minerva Gastroenterol Dietol* 2016;62:88-102.
3. Zucman-Rossi J, Villanueva A, Nault JC, Llovet JM. Genetic Landscape and Biomarkers of Hepatocellular Carcinoma. *Gastroenterology* 2015;149:1226-39.
4. Lombardi A, Grimaldi A, Zappavigna S, Misso G, Caraglia M. Hepatocarcinoma: genetic and epigenetic features. *Minerva Gastroenterol Dietol* 2017 Apr 11. doi: 10.23736/S1121-421X.17.02408-4.
5. European Association For The Study Of The Liver, European Organization For Research And Treatment Of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. *J Hepatol* 2012;56:908–43.
6. Bruix J, Sherman M, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology* 2011;53:1020–2.
7. Arii S, Sata M, Sakamoto M, Shimada M, Kumada T, Shiina S, *et al.* Management of hepatocellular carcinoma: Report of Consensus Meeting in the 45th Annual Meeting of the Japan Society of Hepatology (2009). *Hepatol Res* 2010;40:667–85.
8. Omata M, Lesmana LA, Tateishi R, Chen PJ, Lin SM, Yoshida H, *et al.* Asian Pacific Association for the Study of the Liver consensus recommendations on hepatocellular carcinoma. *Hepatol Int* 2010;4:439-74.
9. Caviglia GP, Abate ML, Petrini E, Gaia S, Rizzetto M, Smedile A. Highly sensitive alpha-fetoprotein, Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein and des-gamma-carboxyprothrombin for hepatocellular carcinoma detection. *Hepatol Res* 2016;46:E130-5.

10. Petrini E, Caviglia GP, Abate ML, Fagoonee S, Smedile A, Pellicano R. MicroRNAs in HBV-related hepatocellular carcinoma: functions and potential clinical applications. *Panminerva Med* 2015;57:201-9.
11. Dong S, Miao AY, Lei W, Chen QW. miR-146a rs2910164 and hepatocellular carcinoma: a meta-analysis. *Minerva Med* 2017;108:287-92.
12. Johnson PJ, Pirrie SJ, Cox TF, Berhane S, Teng M, Palmer D, *et al.* The detection of hepatocellular carcinoma using a prospectively developed and validated model based on serological biomarkers. *Cancer Epidemiol Biomarkers* 2014;23:144–53.
13. El-Serag HB, Kanwal F, Davila JA, Kramer J, Richardson P. A new laboratory-based algorithm to predict development of hepatocellular carcinoma in patients with hepatitis C and cirrhosis. *Gastroenterology* 2014;146:1249-55.
14. Karakatsanis A, Papaconstantinou I, Gazouli M, Lyberopoulou A, Polymeneas G, Voros D. Expression of microRNAs, miR-21, miR-31, miR-122, miR-145, miR-146a, miR-200c, miR-221, miR-222, and miR-223 in patients with hepatocellular carcinoma or intrahepatic cholangiocarcinoma and its prognostic significance. *Mol Carcinog* 2013;52:297-303.
15. Brunetto MR, Cavallone D, Oliveri F, Moriconi F, Colombatto P, Coco B, *et al.* A serum microRNA signature is associated with the immune control of chronic hepatitis B virus infection. *PLoS One*. 2014;9:e110782.
16. Tian YW, Shen Q, Jiang QF, Wang YX, Li K, Xue HZ. Decreased levels of miR-34a and miR-217 act as predictor biomarkers of aggressive progression and poor prognosis in hepatocellular carcinoma. *Minerva Med* 2017;108:108-13.
17. Borel F, Konstantinova P, Jansen PL. Diagnostic and therapeutic potential of miRNA signatures in patients with hepatocellular carcinoma. *J Hepatol* 2012;56:1371-83.

18. Caviglia GP, Abate ML, Petrini E, Gaia S, Manzini P, Carucci P, *et al.* Clinic evaluation of circulating microRNAs as potential biomarkers of hepatocellular carcinoma in patients with HBV chronic infection. *J Hepatol* 2015;62:S447-8.
19. European Association for the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol* 2017 Apr 18. doi: 10.1016/j.jhep.2017.03.021.
20. Caviglia GP, Touscoz GA, Smedile A, Pellicano R. Noninvasive assessment of liver fibrosis: key messages for clinicians. *Pol Arch Med Wewnętrznej* 2014;124:329–35.
21. Bruix J, Reig M, Sherman M. Evidence-Based Diagnosis, Staging, and Treatment of Patients With Hepatocellular Carcinoma. *Gastroenterology* 2016;150:835-53.
22. He S, Hu XW, Wang D, Han LF, Zhang DC, Wei C. Accuracy of microRNAs for the diagnosis of hepatocellular carcinoma: A systematic review and meta-analysis. *Clin Res Hepatol Gastroenterol* 2016;40:405-17.
23. Bandopadhyay M, Sarkar N, Datta S, Das D, Pal A, Panigrahi R, *et al.* Hepatitis B virus X protein mediated suppression of miRNA-122 expression enhances hepatoblastoma cell proliferation through cyclin G1-p53 axis. *Infect Agent Cancer* 2016;11:40.
24. Baek YH, Lee JH, Jang JS, Lee SW, Han JY, Jeong JS, *et al.* Diagnostic role and correlation with staging systems of PIVKA-II compared with AFP. *Hepatogastroenterology* 2009;56:763-7.
25. Seo SI, Kim HS, Kim WJ, Shin WG, Kim DJ, Kim KH, *et al.* Diagnostic value of PIVKA-II and alpha-fetoprotein in hepatitis B virus-associated hepatocellular carcinoma. *World J Gastroenterol* 2015;21:3928-35.
26. Viggiani V, Palombi S, Gennarini G, D'Ettorre G, De Vito C, Angeloni A, *et al.* Protein induced by vitamin K absence or antagonist-II (PIVKA-II) specifically increased in Italian hepatocellular carcinoma patients. *Scand J Gastroenterol.* 2016;51:1257-62.



27. Yu R, Xiang X, Tan Z, Zhou Y, Wang H, Deng G. Efficacy of PIVKA-II in prediction and early detection of hepatocellular carcinoma: a nested case-control study in Chinese patients. *Sci Rep* 2016;6:35050.
28. Sultanik P, Ginguay A, Vandame J, Popovici T, Meritet JF, Cynober L, *et al.* Diagnostic accuracy of des-gamma-carboxy prothrombin for hepatocellular carcinoma in a French cohort using the Lumipulse® G600 analyzer. *J Viral Hepat* 2017;24:80-5.
29. Caviglia GP, Abate ML, Bosco C, Gaia S, Olivero A, Rosso C, *et al.* Cross-sectional and longitudinal evaluation of alpha-fetoprotein (AFP) and protein induced by vitamin k absence or antagonist II (PIVKA-II) in patients with cirrhosis of viral etiology under surveillance for hepatocellular carcinoma (HCC). *Biochim Clin* 2016;40:S124.
30. Feier D, Lupsor Platon M, Stefanescu H, Badea R. Transient elastography for the detection of hepatocellular carcinoma in viral C liver cirrhosis. Is there something else than increased liver stiffness? *J Gastrointestin Liver Dis* 2013;22:283-9.
31. Raffetti E, Fattovich G, Donato F. Incidence of hepatocellular carcinoma in untreated subjects with chronic hepatitis B: a systematic review and meta-analysis. *Liver Int* 2016;36:1239-51.
32. Saracco GM, Evangelista A, Fagoonee S, Ciccone G, Bugianesi E, Caviglia GP, *et al.* Etiology of chronic liver diseases in the Northwest of Italy, 1998 through 2014. *World J Gastroenterol* 2016;22:8187-93.

Table I.-*Demographic and clinical characteristics of the patients included in the cross-sectional study cohort and those included in the longitudinal study cohort at time of HCC diagnosis.*

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCLC, Barcelona Clinic Liver Cancer; CI, confidence interval; F, female; HCC, hepatocellular carcinoma; M, male; PIVKA-II, protein induced by vitamin k absence or antagonist II; Plts, platelets.

Figure 1.-Comparison of miR-122 (A), AFP (B) and PIVKA-II (C) serum levels between patients with cirrhosis and those with HCC.

Abbreviations: AFP, alpha-fetoprotein; AU, arbitrary units; HCC, hepatocellular carcinoma; PIVKA-II, protein induced vitamin k absence or antagonist II.

Figure 2.-ROC curves showing the performance of miR-122, AFP and PIVKA-II alone or in combination for the discrimination of patients with cirrhosis and those with HCC.

The equation obtained from the multiple stepwise logistic regression was:  $\text{Logit}(P) = -2.5336 - 0.0096 (\text{miR-122}) + 0.0215 (\text{AFP}) + 0.0372 (\text{PIVKA-II})$ ; where the probability (P) of HCC in a patient is given by:  $P = 1 / (1 + e^{-\text{Logit}(P)})$ . Abbreviations: AFP, alpha-fetoprotein; AUC, area under the curve; PIVKA-II, protein induced by vitamin k absence or antagonist II.

Figure 3.-Longitudinal variation of miR-122 (A), AFP (B), PIVKA-II (C) and biomarkers combination (C) in the 13 patients with HBV-related cirrhosis that developed HCC.

Friedman test was used to evaluate the kinetics of miR-122, AFP and PIVKA-II alone or in combination, respectively. Error bars represent the 95%CI of the median. Abbreviations: AFP, alpha-fetoprotein; AU, arbitrary units; P(HCC), probability of hepatocellular carcinoma; PIVKA-II, protein induced vitamin k absence or antagonist II.

Figure 4.-Comparison of survival curves between patients with cirrhosis with P(HCC) value  $>0.30$  and those with P-HCC  $\leq 0.30$ .

P-value has been calculated by Log-rank test. Abbreviations: HCC, hepatocellular carcinoma.